

Notes:

- A. For all Qiagen gel extraction steps (Qiaquick and MinElute), melt gel slice at 37° C instead of 50° (see Quail et al 2008 Nature Methods). Incubate for 30 min, vortexing frequently - make sure gel is completely melted before proceeding.
- B. For Qiagen Qiaquick and MinElute column purifications (not gel extractions) use Qiagen buffer PB, not PBI (newer Qiagen kits don't even have PBI).
- C. For size-selection after adapter ligation, be aware that the adapters are only partially double-stranded so adapter-ligated fragments might run at slightly different position than expected.
- D. All gel purifications are performed with TAE agarose gels. We add 1:10000 Sybr-Safe to gel after microwaving (instead of Et Br).
- E. The Illumina Genomic DNA Sample kit manual specifies Bio-Rad certified low-range Ultra Agarose for gel extraction steps; the ChIP-seq Sample kit manual does not specify a particular agarose. I have been using the Bio-Rad Ultra Agarose.
- F. The minimum amount of DNA needed for each experiment is currently 2 ug (starting amount for sonication), but it might be possible to decrease.

PROTOCOL:

1. Start with high-quality, high molecular weight gDNA (integrity of DNA is critical if it will also be used for MRE-seq). Isolate DNA using the Costello lab's "genomic DNA extraction from frozen tissue" protocol. Include overnight proteinase K digestion and follow with a 1 hour, 37 degrees DNase-free RNase treatment (Roche catalog# 11 119 915 001) (final RNase concentration = 40 µg/ml). Perform 2 PCI and 2 chloroform extractions using 2 ml "Light" phase lock gels. Be careful at all steps not to shear DNA – always use wide-bore tips and pipet gently. Resuspend DNA in TE (not water). Store DNA at 4° C.
2. Sonicate 50 – 150 ul of DNA (at 100 ng/ul in TE) in microfuge tube with Diagenode Bioruptor Sonicator. If DNA amount is limiting, can sonicate using lower DNA concentration. Sonicate 15 seconds on/45 seconds off. Depending on how intact the DNA is, sonicate between 20-50 pulses. Change water bath and add fresh ice periodically to keep cool. Verify fragment size on 1% agarose gel: want ~ 100 – 400 bp.
3. Qiagen Qiaquick purify sonicated DNA (to concentrate and have in EB instead of TE). Elute in 30 µl EB. Check concentration with nanodrop. Set aside an aliquot for input control.
4. Repair reaction. Follow the standard Illumina repair protocol, starting with 2 µg DNA:

Illumina ultrapure water	45 µl
sonicated DNA (~ 2 ug)	30
T4 DNA ligase buffer with 10mM ATP	10
dNTPs	4
Klenow DNA polymerase	1
T4 DNA polymerase	5
T4 PNK	<u>5</u>
total volume	100

incubate in thermal cycler 30 min at 20°C

5. Clean up reaction with Qiagen Qiaquick column, as per Qiagen instructions. Elute in 32 µl EB.
6. Addition of 3' A (using Illumina reagents)

DNA sample	32 μ l
Klenow buffer	5
dATP	10
Klenow 3'-5' exo minus	<u>3</u>
total	50

incubate in thermal cycler 30 min at 37°C

7. Clean up with Qiagen MinElute column, elute in 10 μ l EB.

8. Adapter Ligation. Follow the Illumina Genomic DNA kit protocol, which uses 10 μ l adapter oligo mix for 5 μ g starting DNA.

DNA	10 μ l
Illumina DNA ligase buffer	25
Illumina adapter oligo mix	10
Illumina DNA ligase	<u>5</u>
total	50

incubate 15 min at room temperature

9. Clean up with Qiagen Qiaquick column, elute in 30 μ l EB.

10. Size-select adapter-ligated fragments. Make a 50 ml, 2% TAE gel (Owl small gel; use thick 10-well comb). Mix 30 μ l DNA with 10 μ l 4X tris/EDTA/sucrose loading buffer (does not contain dyes). Loading gel: skip first and last wells. Load all 40 μ l sample on one side of gel; load 10 μ l of 100 bp ladder and 5 μ l of NEB Low MW ladder on other side of gel. Load a lane next to markers with 0.5 μ l dye only (BPB and XC) to help monitor gel running. Run at 120 V 1 hr. Size range to excise 166 to 366 bp. Gel-purify each with a single Qiagen Qiaquick column (for gel slices < 400 mg) or two or more Qiagen MinElute columns (for gel slices > 400 mg). Elute in 20 μ l EB.

11. Use 2 μ l for Nanodrop, store rest at -20 until MeDIP.

MeDIP Reagents.

A. sodium phosphate buffer (note: phosphate buffers will become contaminated with microorganisms over time. Make fresh stocks every 2 months, and use working 0.1 buffer within ~ 1 week, storing at 4 degrees)

0.2 M dibasic sodium phosphate stock solution:

dibasic sodium phosphate, anhydrous (Na_2HPO_4) (MW = 141.96) : 28.4 g
volume to 1 L with ddH₂O
autoclave or filter sterilize

0.2 M monobasic sodium phosphate stock solution:

monobasic sodium phosphate, dihydrate ($\text{NaH}_2\text{PO}_4 + 2\text{H}_2\text{O}$) (MW = 155.99) : 31.2 g
volume to 1 L with ddH₂O
autoclave or filter sterilize

0.1 M sodium phosphate buffer, pH 7.0:

0.2 M dibasic sodium phosphate stock solution: 30.5 ml
0.2 M monobasic sodium phosphate stock solution: 19.5 ml
autoclaved ddH₂O: 50 ml

100 ml

verify pH=7.0 with indicator paper

B. 2M NaCl

NaCl: 5.84 g
volume to 50 ml with ddH₂O
autoclave or filter sterilize

C. 10% Triton X-100

D. IP wash buffer

autoclaved ddH ₂ O	82.5 ml	
0.1 M sodium phosphate buffer, pH 7.0	10.0 ml	Final concentration 10 μ M
2M NaCl	7.0 ml	Final concentration 140 mM
10% triton X-100	<u>500 μl</u>	Final 0.05%
	100 ml	

E. anti-methylcytosine antibody: Eurogentec catalog # BI-MECY-0100 (mouse monoclonal IgG₁) concentration 1 mg/ml in PBS

F. Rabbit anti-mouse IgG (affinity purified)
Jackson Immunoresearch catalog # 315-005-008
Check each lot for concentration (current one is 2.4 mg/ml)

G. Protein A/G beads
Pierce catalog # 20421

H. Optional : Negative control antibody : non-specific mouse IgG
Jackson Immunoresearch catalog # 015-000-003
Check each lot for concentration (current one is 5.5 mg/ml)

12. MeDIP denaturation. Pipet volume of DNA corresponding to 500 ng - 1 μ g into 0.2 ml PCR tube. Denature DNA at 95° C for 10 minutes. Transfer immediately to ice to prevent re-annealing. Keep on ice for 10 min. Spin briefly.

13. MeDIP IP setup (use autoclaved 1.5 ml screwcap tubes):

sonicated, denatured DNA:	_____ μ l (for 500 ng - 1 μ g)
0.1 M sodium phosphate buffer, pH 7.0	50 μ l
2M NaCl	35 μ l
10% triton X-100	2.5 μ l
anti-methylcytidine primary antibody(1mg/ml)	0.5 - 1 μ l (1 μ g antibody/1 μ g DNA)
autoclaved ddH ₂ O	to 500 μ l

incubate at 4° C overnight (16 hrs) on rotator

14. add secondary antibody and beads:

Rabbit anti-mouse IgG (Fc fragment specific) antibody, ~2.5 μ g/ μ l:	1 μ l
Protein A/G beads (***USE WIDE-ORIFICE FILTERED TIPS):	100 μ l

incubate at 4° C for 2 hours on rotator

During incubation, make proteinase K solution:

TE:	962.5 µl
proteinase K, 20 mg/ml stock:	12.5 µl
10% SDS	<u>25 µl</u>
	1000 µl

15. Spin tubes at 2000 g for 2 minutes in 4° microfuge (all wash steps should be done with 4° microfuge). Carefully transfer supernatant to a normal microfuge tube using a p1000 and save if desired. Otherwise discard supernatant.

16. Resuspend pellet in 1000 µl cold IP wash buffer and transfer to a normal microfuge tube. Make sure that pellet is fully resuspended.

17. Spin at 2000 g for 1 minute. Carefully discard supernatant using p1000.

18. Repeat steps 7 – 8 five times (for a total of 6 washes). For each wash, make sure that pellet is fully resuspended.

19. For final spin, centrifuge at 5000 g for 2 minutes. Remove and discard supernatant. Re-centrifuge at 5000 g for 2 minutes; remove all remaining liquid with a filtered gel-loading pipet tip.

20. Add 200 µl of SDS/proteinase K solution to each pellet. Resuspend thoroughly.

21. Incubate at 50° for 2 hours, mixing occasionally. Cool to RT.

22. Purify DNA from IPs (and supernatants, if desired) using Qiagen Qiaquick Kit. Elute MeDIP DNA in 30 µl Qiagen EB buffer. Expect about 10% yield.

23. Illumina PCR of adapter-modified, MeDIP-enriched fragments. Use 1/3 of IP'd DNA as template.

DNA	10
Illumina Phusion DNA polymerase	25
Illumina PE PCR primer forward	1
Illumina PE PCR primer reverse	1
Illumina Ultrapure water	<u>13</u>
total	50

cycling conditions:

30 sec 98 °C
15 (or less) cycles of:
10 sec 98 °C
30 sec 65 °C
30 sec 72 °C
Then: 5 min 72 °C
Then: hold at 4 °C

14. Size select (220-420 bp for PE) by Qiagen MinElute gel purification. Elute in 20 µl EB (2 columns, 10 ul for each).

15. PCR confirmation of MeDIP enrichment. Dilute an aliquot of each library to 5 ng/ul in EB. Compare to 5 ng/ul of input.

Pr 3/4 – CpG-less sequence (~ 1kb) on Ch15 (negative control; 207 bp)
(F: CCTGCAACTTTACTGAATTTAGC; R: GGAATCTCACTTTCACCACTGG)

Pr 7/8 -- SNRPN 5' end (methylated on one allele) (positive control; 156 bp)
(F:CGCTCAACACCCCCTAAATA; R: GGTGGAGGTGGGTACATCAG)

Pr 9/10 – MAGEA1 promoter methylated in normal cells on X ch (positive control; 105 bp)
(F:GTTCCCGCCAGGAAACATC; R: GAACTCTACGCCGTCCCTCAG)

Pr 11/12 – GAPDH promoter unmethylated on both alleles (negative control; 112 bp)
(F: CGTAGCTCAGGCCTCAAGAC; R: GCTGCGGGCTCAATTTATAG)

PCR setup:

	<u>1X</u>
H2O	13.9
Qiagen 10X CoralLoad PCR buffer	2
Qiagen dNTPs	0.4
Forward primer (10 µM)	0.8
Reverse primer (10 µM)	0.8
DNA (5 ng/ul)	2
Qiagen taq	<u>0.1</u>
	20

Cycling: 95° 5:00

29 cycles of:

95° 0:30

58° 0:30

72° 0:30

then

72° 5:00

6° ∞

Gel: Run 10 ul of each on 1.8% agarose

Sequencing:

Dilute purified library to 10nmol, load 10-12pmol for cluster generation